Substitution of Lysine at Position 104 or 240 of TEM-1_{pTZ18R} β-Lactamase Enhances the Effect of Serine-164 Substitution on Hydrolysis or Affinity for Cephalosporins and the Monobactam Aztreonam

Judith A. Sowek,[‡] Susan B. Singer,[§] Shari Ohringer,[§] Mary F. Malley,[§] Thomas J. Dougherty,[‡] Jack Z. Gougoutas,[§] and Karen Bush*,[§]

Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 5100, Wallingford, Connecticut 06492, and P.O. Box 4000, Princeton, New Jersey 08543

Received July 26, 1990; Revised Manuscript Received November 11, 1990

ABSTRACT: By site-directed mutagenesis, TEM-1 β-lactamase was altered to contain single amino acid changes of E104K, R164S, and E240K, in addition to double changes of E104K/R164S or R164S/E240K and the triple change of E104K/R164S/E240K. Hydrolysis rates for cephaloridine and benzylpenicillin were lowered at least 1 order of magnitude for all enzymes containing R164S substitutions. All mutant enzymes exhibited increased k_{cat} values for β-lactam antibiotics containing an aminothiazole oxime side chain. Hydrolysis of ceftazidime was most affected, with k_{cat} values increased 3–4 orders of magnitude in all enzymes with the substituted R164S moiety. K_m values decreased for all substrates except ceftazidime in the enzymes with multiple mutations. Aztreonam was most affected, with K_m values lowered 23–56-fold in the enzymes bearing multiple mutations. When the crystal structures of aztreonam and related monobactams were studied and projected into an active-site model of the PC1 β-lactamase, it became apparent that the two lysine residues might serve equivalent roles by interacting with the carboxylate of the aminothiazole oxime side chain. Hydrogen-bonding interactions involving the oxime and N7 of the lysine, particularly Lys-104, may also be important in some antibiotics. Ser-164 apparently serves an indirect role, since it is somewhat distant from the active-site cleft.

Hydrolysis of β-lactam antibiotics by enzymes known as β-lactamases represents the most common mechanism by which bacteria exhibit resistance to this group of therapeutic agents. The TEM-1 β-lactamase is the most common plasmid-mediated enzyme of this class and has been shown to comprise as much as 72% of the population of β-lactamases that have been identified in penicillin-resistant Gram-negative bacteria (Medeiros, 1984). Although many β-lactam antibiotics were developed to exhibit exquisite stability to this enzyme, over the past 5 years clinicians have identified many naturally occurring TEM-related enzymes capable of hydrolyzing previously stable extended-spectrum antibiotics such as aztreonam and ceftazidime (Chanal et al., 1989; Philippon et al., 1989). When amino acid sequences of the natural enzymes were deduced from nucleotide sequences, common substitutions in critical areas were recognized, in addition to some possibly superfluous substitutions (Table I). It should be noted that the TEM-2 β-lactamase was observed many years ago to exhibit no biological characteristics different from those of TEM-1; the sequences of these enzymes differ by a single amino acid at position 39.

Certain substitutions in the more recently identified enzymes have been suggested to be responsible for the observed increased hydrolytic characteristics, although none of these amino acids were previously implicated as an active-site residue. However, many of these residues are adjacent to conserved sequences among the class A β-lactamases (Joris et al., 1988) and have been proposed to be adjoining the active-site crevice. In particular, substitution of either lysine-104 or serine-164 was present in each of the enzymes capable of hydrolyzing extended-spectrum antibiotics (TEM-3–TEM-7). Another commonly substituted amino acid, serine-238, has been suggested to interact with the oxime on the cephalosporin or monobactam side chain (Labia et al., 1988; Philippon et al., 1989), whereas the ε-amino group of lysine-104 or lysine-240 may interact with a negative group on the ceftazidime side chain, perhaps through formation of a salt bond with the acidic substituent (Peduzzi et al., 1989).

Site-directed mutagenesis of these enzymes to determine the critical substitutions appears to be a logical extension of the results obtained from nature. Several studies have been reported in this area, with all possible natural amino acids substituted at position 71 adjacent to the active-site serine (Schultz & Richards, 1986) and at residue 237 (Healey et al., 1989). Other genetic studies have examined nonspecific mutations (Dube & Loeb, 1989), including an early description of a more active mutant enzyme selected after chemical mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Hall & Knowles, 1976), an enzyme now described as an alanine-237 mutant (Healey et al., 1989). Specific enzymes constructed genetically include a TEM-1 derivative with substitutions of lysine for glutamic acid at position 104 and serine for glycine at 238 (Sougakoff et al., 1988). However, none of these studies included rigorous biochemical descriptions of the mutant enzymes. Constructions of specific mutant TEM enzymes with modifications centered at the active-site serine have been described by Sigal et al. (1982) and Dalbadie-McFarland et al. (1982). A recent study more completely described the catalytic role of Lys-234 by the use of site-directed mutagenesis of the *Bacillus licheniformis* β-lactamase, a class A β-lactamase like the TEM enzymes (Ellerby et al., 1990).

In this work we describe a set of TEM-1 derivatives that have been constructed through site-directed mutagenesis to study sequential changes in amino acid composition. Single-step mutations based upon modified residues in naturally occurring TEM variants were introduced to determine the exact contributions of each substitution on both the catalytic

[‡] Wallingford, CT.
[§] Princeton, NJ.

0006-2960/91/0430-3179$02.50/0

Table I: Amino Acid Modifications Identified in Naturally Occurring TEM β-Lactamases

enzyme[a]	residue 21	39	104	164	237	238[b]	240	265	reference
TEM-1_{pBR322}	Leu	Gln	Glu	Arg	Ala	Gly	Glu	Thr	Sutcliffe, 1978
TEM-2_{R6K}	Leu	LYS[c]	Glu	Arg	Ala	Gly	Glu	Thr	Ambler & Scott, 1978
TEM-3_{pCF04}	Leu	LYS	LYS	Arg	Ala	SER	Glu	Thr	Sougakoff et al., 1988
TEM-4_{pUD16}	PHE	Gln	LYS	Arg	Ala	SER	Glu	MET	Sougakoff et al., 1989
TEM-5_{pCFF14}	Leu	Gln	Glu	SER	THR	Gly	LYS	Thr	Sougakoff et al., 1989
TEM-6	Leu	Gln	LYS	HIS	Ala	Gly	Glu	Thr	Philippon et al., 1989
TEM-7_{pIF100}	Leu	LYS	Glu	SER	Ala	Gly	Glu	Thr	Collatz et al., 1989
TEM-9	PHE	Gln	LYS	SER	Ala	Gly	Glu	MET	Mabilat et al., 1990

[a] Subscripts indicate plasmid from which sequence was determined. [b] Note that residues 238 and 240 are adjacent in the TEM sequences. The numbering of amino acids in TEM β-lactamases is not consecutive due to insertions in β-lactamase sequences of homologous class A enzymes. [c] Amino acids in capital letters indicate changes from the TEM-1 enzyme, with numbering according to Ambler (1979).

rate and the affinity for selected substrates. The working hypothesis of this study was that substitution of serine at position 164 would affect catalytic activity to a lesser extent than the combination of a lysine/serine change. We also postulated that lysine substitutions at either position 104 or 240 might serve equivalent functions—to interact with an acidic group on the side chain of antibiotics with aminothiazole oxime substituents. A triple mutant coding for an enzyme with both substituted lysines in combination with serine-164 was also constructed to determine the effect upon enzymatic activity.

As we examined the systematic substitutions in TEM-1 β-lactamase, we determined kinetic parameters for hydrolysis of selected substrates. In particular, the hydrolysis of aztreonam and ceftazidime, two β-lactam antibiotics that contain the same acidic aminothiazole oxime side chain, was contrasted to the hydrolysis of cefotaxime, a similar cephalosporin with a neutral side chain (Figure 1). Using the crystal structure of aztreonam, we have proposed possible interactions between this substrate and the PC1 penicillinase, a class A β-lactamase, on the basis of the published crystal structure of that enzyme (Herzberg & Moult, 1987).

Experimental Procedures

Materials. Antibiotics were obtained from the following sources: cephaloridine from Eli Lilly and Co. (Indianapolis, IN); benzylpenicillin and aztreonam and other monobactams from Bristol-Myers Squibb (Princeton, NJ); clavulanic acid from Beecham Laboratories (Bristol, TN); ceftazidime from Glaxo (Greenford, U.K.); cefotaxime from Hoechst-Roussel (Somerville, NJ). Sigma Chemical Co. (St. Louis, MO) was the supplier for 6-aminohexanoic acid activated Sepharose 4B, *m*-aminophenylboronic acid, triethanolamine hydrochloride, and boric acid.

Bacterial strains used for mutagenesis included a dUTPase- and uracil-*N*-glycosylase-lacking *Escherichia coli* mutant (CJ236 dut$^-$, ung$^-$ from B. Bachman, CGSC, New Haven, CT) and *E. coli* JM109 (Pharmacia, Piscataway, NJ).

Single-Stranded DNA Production and Isolation. The phagemid pTZ18R (Pharmacia) containing a β-lactamase gene and the f1 intergenic region can readily generate single-stranded DNA (ssDNA) in the presence of a helper phage. Uracil-incorporated ss pTZ18R DNA was utilized as the template for mutagenesis. A 5.0-mL aliquot of a midlog culture of F$^+$ CJ236 transformed with the phagemid pTZ18R was inoculated into 100 mL of 2X YT broth (tryptone, 16 g/L; yeast extract, 10 g/L; NaCl, 10 g/L; pH 7.4) supplemented with 0.25 mg/L uridine and 50 mg/L ampicillin. The cultures were incubated at 35 °C for 30 min with vigorous shaking (300 rpm), followed by the addition of M13KO7 helper phage (kanamycin resistant) at 1×10^8 plaque-forming units. The incubation was continued for an additional 30 min followed by the addition of kanamycin at 70 mg/L. The final incubation for phagemid extrusion into the media continued for 14–18 h. Isolation of uracil-containing single-stranded pTZ18R DNA was carried out as described by Kunkel (1987).

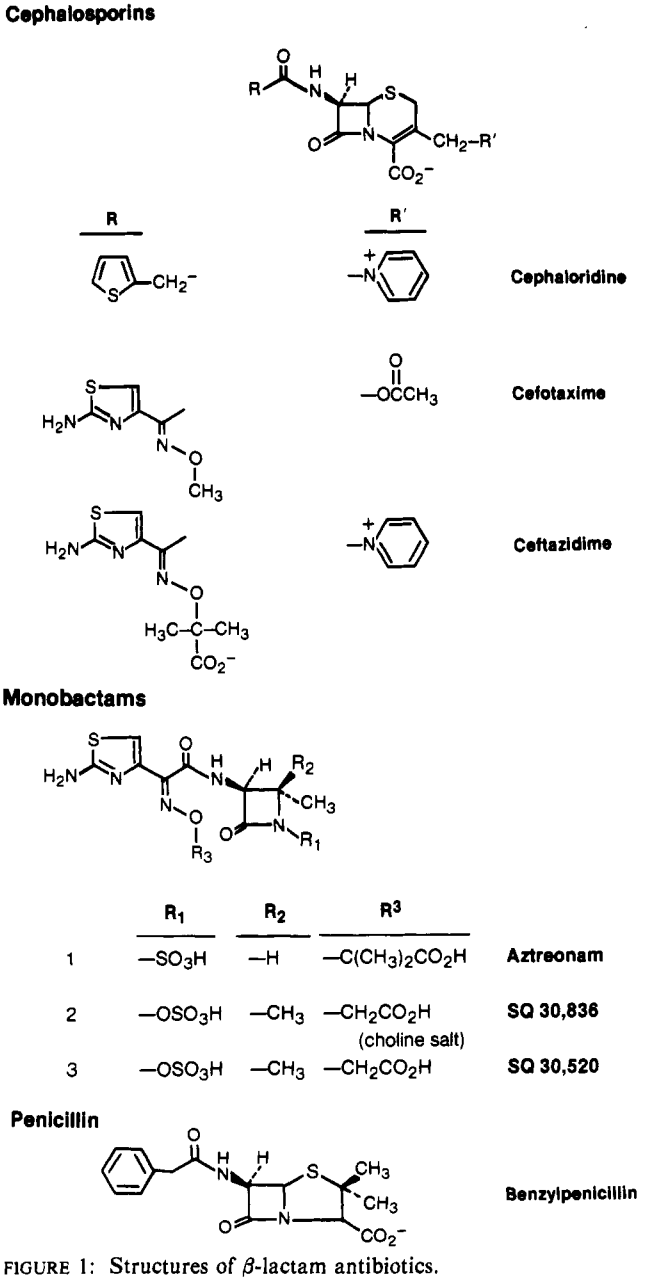

FIGURE 1: Structures of β-lactam antibiotics.

Mutagenesis and Selection of Isolates. Mutagenesis of the pTZ18R β-lactamase gene was carried out by priming with synthetic oligonucleotides (Synthetic Genetics, San Diego, CA)

containing the desired mutation. After oligonucleotide hybridization to the ss template, primer extension was performed essentially as described by Kunkel (1987). Resultant plasmids were transformed into competent (CaCl treated) JM109 for primary dut^+, ung^+ selection of the mutant strand as previously described (Kunkel, 1987). Ampicillin-resistant colonies were selected, and determinations of MIC (minimal inhibitory concentration) were carried out for aztreonam, cefotaxime, and ceftazidime. Isolates with MICs elevated for any of the three antibiotics relative to JM109 containing the parent pTZ18R were chosen for DNA sequencing.

DNA Sequencing. Dideoxy chain termination sequencing reactions were carried out with ssDNA template, isolated as described above, by using ^{35}S-ATP (New England Nuclear, Boston, MA). A T^7 sequencing kit (Pharmacia) was used according to the manufacturer's directions. When necessary, deoxyinosine triphosphate was utilized to eliminate band compression. Sequencing gels were run on a BRL Model S2 apparatus. The parent pTZ18R β-lactamase gene and all mutant genes were sequenced in entirety.

Microbiological Activity. MIC determinations were performed by agar dilution on Mueller–Hinton agar with an inoculum of 10^4 colony-forming units/spot (National Committee for Clinical Laboratory Standards).

β-Lactamase Purification. All β-lactamases were purified to >90% homogeneity as follows. Enzyme-producing organisms were grown in trypticase soy broth and harvested during log phase. Cells were broken by a freeze-thaw procedure (Bush & Singer, 1989), and the clarified supernatant was eluted from Sephadex G-75 in 0.05 M phosphate buffer, pH 7.0 (Quinn et al., 1989). Fractions containing β-lactamase activity were pooled and dialyzed overnight against 20 mM triethanolamine/0.5 M NaCl, pH 7.0. The β-lactamase activity was then eluted from a type B aminophenylboronic acid–agarose column (Cartwright & Waley, 1984) that had been equilibrated with the same buffer used for dialysis. Protein with minimal β-lactamase activity eluted initially. The major peak of β-lactamase activity was usually obtained immediately after the buffer was changed to 0.5 M borate/0.5 M NaCl, pH 7.0. However, the enzymes resulting from a single lysine substitution, E104K and E240K, often eluted as double peaks from the boronic acid column. The second peak yielded an enzyme with higher specific activity and was used for the kinetic studies as described below. Active fractions from each type B column purification were pooled and dialyzed overnight against 0.05 M phosphate buffer, pH 7.0, with three buffer changes. This preparation was stored at 4 °C in the presence of 0.02% sodium azide and used for determinations of kinetic constants. The enzyme bearing the triple mutation exhibited the greatest instability. When the main fraction of activity from the Sephadex column was submitted to affinity chromotography and dialysis, the enzyme activity degraded into a doublet as determined by isoelectric focusing, perhaps due to copurification of a small amount of protease activity that could act upon the mutant enzyme. When the leading and tailing fractions from the Sephadex purification were submitted to phenylboronic acid–agarose chromatography followed by dialysis, at least 90% of the β-lactamase activity was associated with a single band of p*I* 6.25 on analytical isoelectric focusing. This latter preparation was used immediately for kinetic studies.

Isoelectric Focusing. Isoelectric focusing was performed with an LKB Multiphor with prepared PAG plates, pH range of 3.5–9.5 or 4.0–6.5. Gels were stained for activity with the chromogenic cephalosporin SQ 24902 and for protein with silver stain (Bio-Rad, Richmond, CA).

Polyacrylamide Gel Electrophoresis. PAGE was performed as described by Laemmli (1970) in the absence or presence of SDS. Precast gels were obtained from ISS-Enprotech (Hyde Park, MA). Gels were stained for β-lactamase activity with SQ 24902, a chromogenic cephalosporin. Protein staining was performed with silver stain.

β-Lactamase Assays. Hydrolysis of β-lactam antibiotics was determined spectrophotometrically at 25 °C in 0.1 M phosphate buffer, pH 7.0, on a Gilford 250 or 2600 spectrophotometer. Spectral parameters for cephaloridine, benzylpenicillin, cefotaxime, ceftazidime, and aztreonam have been described previously (Quinn et al., 1989). Kinetic parameters of V_{max} and K_m were determined in at least duplicate experiments by the four kinetic analyses in the program ENZPACK (Elsevier, Biosoft.)

Inhibition by clavulanic acid was determined by incubating enzyme with buffer or varying concentrations of inhibitor (1.0–2000 nM) in a volume of 70–150 μL for 5 min at 25 °C before the addition of cephaloridine, to give a final cephaloridine concentration of 1.0 mM in 600 μL. I_{50} values were calculated graphically to give the concentration of inhibitor required to lower the activity of the control enzyme by 50%. Reported values are based on at least two sets of determinations conducted on different days.

Crystal Structure Analysis. For all analyses unit cell dimensions were obtained from least-squares analysis of the angular diffractometer (Enraf-Nonius CAD-4) settings of 25 reflections (2θ = 40–50°). Intensities were measured at 23 °C with the θ–2θ variable scan technique (Cu Kα) and corrected only for Lorentz–polarization factors; background counts were measured at the extremes of the scans for half the times of the scans. The structures were solved by direct methods and refined by full-matrix least-squares analysis based on *F*, with the SDP software package (*Structure Determination Package*, B. A. Frenz and Associates, College Station, TX 77804). No crystal decomposition was observed during data collection.

Estimation of the Three-Dimensional Structure of PC1 β-Lactamase. The three-dimensional atomic coordinates of the backbone and side-chain atoms of the active-site residues of the PC1 β-lactamase were estimated from the measured parallax in published stereodrawings [Figures 1 and 4 of Herzberg and Moult (1987)], by a locally modified version of Rossmann's computer program STEREO (Rossmann & Argos, 1980; Brookhaven Protein Databank Software distribution, 1979). In our experience, this algorithm provides a good estimate of the three-dimensional structure of small molecules and local domains of larger structures but often results in systematic distortions across large (>10-Å) distances. We therefore digitized several overlapping small domains (two to five residues), in order to obtain the local three-dimensional orientations of backbone and side-chain bonds. Idealized adjustments were made to some bond distances and angles in order to correct obvious errors of digitizing the *xy* coordinates of atoms that were nearly coincident in the projected drawings. In all cases, the derived positions of the Cα atoms were adjusted to exact coincidence with the crystallographically derived experimental coordinates deposited in the Brookhaven Protein Database (REFCODE = 1BLM).

RESULTS

Description of Enzymes. The β-lactamase designated TEM-1_{pTZ18R} has been identified as a TEM-1 derivative that has two amino acid changes as a result of separate point mutations in the pBR322 gene: V82I and A182V. These two

Table II: Characteristics of Mutant β-Lactamases

	plasmid						
	pTZ18R	pRDD002	pRDD003	pRDD004	pRDD005	pRDD006	pRD007
nucleotide modified							
304	GAG	AAG	GAG	GAG	AAG	GAG	AAG
484	CGT	CGT	CGT	AGT	AGT	AGT	AGT
709	GAG	GAG	AAG	GAG	GAG	AAG	AAG
enzyme produced	TEM-1_{pTZ}	E104K	E240K	R164S	E104K/R164S	R164S/E240K	E104K/R164S/E240K
amino acid position modified							
104	Glu	LYS	Glu	Glu	LYS	Glu	LYS
164	Arg	Arg	Arg	SER	SER	SER	SER
240	Glu	Glu	LYS	Glu	Glu	LYS	LYS
isoelectric point	5.40	5.95	5.95	5.20	5.63	5.63	6.25

Table III: Microbiological Activities of β-Lactam Antibiotics with Organisms Producing Mutant β-Lactamases

	MIC[a] for plasmid-containing *E. coli* JM109						
plasmid	pTZ18R	pRDD002	pRDD003	pRDD004	pRDD005	pRDD006	pRDD007
enzyme produced	pTZ18R	E104K	E240K	R164S	E104K/R164S	R164S/E240K	E104K/R164S/E240K
antibiotic							
cephaloridine	128	32	128	16	8.0	16	16
cefotaxime	0.03	0.03	0.03	0.03	0.25	0.25	0.13
ceftazidime	0.25	0.25	0.5	2.0	64	32	256
aztreonam	0.06	0.06	0.06	0.06	8.0	16	64
benzylpenicillin	>128	>128	>128	>128	>128	>128	>128

[a] Minimum inhibitory concentration in micrograms per milliliter.

Table IV: Hydrolytic Characteristics of Mutant β-Lactamases

	enzyme						
	TEM-1_{pTZ}	E104K	E240K	R164S	E104K/R164S	R164S/E240K	E104K/R164S/E240K
k_{cat} (s^{-1})							
cephaloridine	750	1240	480	46	30	8.4	19
cefotaxime	0.25	2.5	0.66	2.4	2.0	0.39	0.48
ceftazidime	0.0016	0.067	0.28	3.4	57	17	6.5
aztreonam	0.37	4.4	3.2	5.3	10	2.2	4.0
benzylpenicillin	520	370	410	54	32	17	10
relative k_{cat}							
cephaloridine	1.0	1.7	0.64	0.061	0.040	0.011	0.025
cefotaxime	1.0	10	2.6	9.6	8.0	1.6	1.9
ceftazidime	1.0	42	180	2100	36000	11000	4000
aztreonam	1.0	12	8.6	14	27	5.9	11
benzylpenicillin	1.0	0.71	0.79	0.10	0.062	0.033	0.019

conservative changes have not appeared to alter the biochemical, or physical, properties of the enzyme compared to TEM-1_{pBR322}. Isoelectric points and kinetic parameters for the substrates in this study remained indistinguishable between the TEM-1_{pBR322} and TEM-1_{pTZ18R} enzymes.

Each of the TEM-derived enzymes in this study was the result of stepwise mutations introduced into a TEM β-lactamase (Table II). The enzymes produced by these mutations exhibited physical characteristics similar to those that would be predicted. For example, isoelectric points of enzymes with single lysine substitutions were equivalent, as were the p*I*s of the enzymes with double mutations. The changes observed were consistent with the amino acid changes: lysine substituted for glutamic acid yielded a more basic enzyme; serine for arginine resulted in an enzyme with a lower p*I* than the parent.

All enzymes were purified to greater than 90% homogeneity as determined by isoelectric focusing. The two mutant enzymes resulting from a single lysine substitution appeared to be less stable than others in this study, with satellite β-lactamase bands in each case appearing within 2 days after preparation of the purified enzymes. However, the enzyme bearing the triple mutation was the most unstable. Because of questions of stability, most studies were performed within 1 day of the final purification step.

Molecular weights of the mutant enzymes were approximately 29 000 on SDS–PAGE, very similar to that determined for the TEM-1_{pBR322} enzyme. Small molecular size differences were observed in a nondenaturing gel system, suggesting different molecular shapes for the mutant enzymes.

Microbiological Activity. The plasmid-transformed *E. coli* strains producing the mutant enzymes were tested for susceptibility to the selected group of antibiotics in this study. As seen in Table III, none of the organisms were well inhibited by cephaloridine or benzylpenicillin, standard reference β-lactam antibiotics known to be good substrates for TEM β-lactamases (Bush, 1989). The extended-spectrum antibiotics ceftazidime, cefotaxime, and aztreonam were all effective at inhibiting growth of strains containing plasmids pTZ18R, pRDD002, and pRDD003. Ceftazidime was less active against the strain with pRDD004. Highest resistance was observed for organisms producing enzymes with double mutations, although cefotaxime was still quite active. MICs of aztreonam were the same as cephaloridine for the double mutants but were elevated for the triple mutant. Ceftazidime, with the highest MICs, was the least active cephalosporin against these organisms.

Steady-State Kinetics. Hydrolysis studies for each of these antibiotics resulted in the k_{cat} values shown in Table IV.

Table V: Binding Parameters for Mutant β-Lactamases

	enzyme						
	TEM-1_{pTZ}	E104K	E240K	R164S	E104K/ R164S	R164S/ E240K	E104K/ R164S/ E240K
K_m (μM)							
cephaloridine	660	660	380	100	90	40	70
cefotaxime	450	470	140	230	84	32	57
ceftazidime	80	150	460	260	150	190	56
aztreonam	1500	1400	1600	1400	64	27	43
benzylpenicillin	26	19	31	16	5.8	5.7	14
relative affinity							
cephaloridine	1.0	1.0	1.7	6.6	7.3	17	9.4
cefotaxime	1.0	0.95	3.2	2.0	5.3	14	8.0
ceftazidime	1.0	0.53	0.17	0.31	0.53	0.42	1.4
aztreonam	1.0	1.1	0.94	1.1	23	56	35
benzylpenicillin	1.0	1.4	0.84	1.6	4.5	4.6	1.9
I_{50}[a] (nM)							
clavulanic acid	90	30	84	18	6.2	9.5	12

[a] Enzyme and inhibitor were incubated 5.0 min at 25 °C before the reaction was initiated by addition of cephaloridine at 1.0 mM.

Table VI: Catalytic Efficiency of Mutant β-Lactamases

	enzyme						
	TEM-1_{pTZ}	E104K	E240K	R164S	E104K/ R164S	R164S/ E240K	E104K/ R164S/ E240K
k_{cat}/K_m (s^{-1} mM^{-1})							
cephaloridine	1100	1900	1300	460	330	210	250
cefotaxime	0.56	5.3	4.7	10	24	12	12
ceftazidime	0.02	0.45	0.61	13	380	89	120
aztreonam	0.25	3.1	2.0	3.8	160	81	94
benzylpenicillin	20000	19000	13000	3400	5500	3000	750
relative k_{cat}/K_m							
cephaloridine	1.0	1.7	1.2	0.42	0.30	0.19	0.23
cefotaxime	1.0	9.5	8.4	18	43	21	21
ceftazidime	1.0	23	31	650	19000	4500	6000
aztreonam	1.0	12	8.0	15	640	320	380
benzylpenicillin	1.0	0.95	0.65	0.17	0.28	0.15	0.038

Hydrolysis of the reference antibiotics was affected less than 2-fold by single lysine substitutions. Substitution of serine at position 164 resulted in cephaloridine and benzylpenicillin hydrolysis rates that were lowered at least 1 order of magnitude. Values for k_{cat} were decreased 20–70-fold for the three enzymes with combined lysine/serine substitutions compared to the parent pTZ18 enzyme.

In contrast, hydrolysis of cefotaxime and aztreonam increased moderately in the three enzymes bearing single mutations. The three enzymes containing Lys-240 hydrolyzed cefotaxime with lower k_{cat} values than enzymes with any of the other substitutions. In all the mutant enzymes aztreonam hydrolysis proceeded faster than cefotaxime hydrolysis.

Hydrolysis of ceftazidime was affected dramatically with all the mutations. Increases up to 180-fold were observed in the two lysine-substituted enzymes, with k_{cat} increased 3 orders of magnitude in the R164K enzyme. Hydrolysis rates were increased at least 4 orders of magnitude in the enzymes with double mutations. However, the absolute values for k_{cat} for ceftazidime equaled or exceeded those for cephaloridine and benzylpencillin only in the enzymes with double mutations. The enzyme resulting from the triple mutation hydrolyzed ceftazidime slower than either enzyme with double mutations, but faster than any of the enzymes with single changes.

Affinities for the mutant enzymes showed similar trends for the substrates cephaloridine, cefotaxime, and benzylpenicillin (Table V). Each bound to E104K and E240K, enzymes with single lysine substitutions, with K_m values comparable to that observed for the parent enzyme. Binding to the enzymes with the double or triple mutations was tighter for these antibiotics. In contrast, K_m values for ceftazidime were elevated for all the mutant enzymes compared to the parent TEM-1 except for the enzyme bearing the triple substitution. Binding of aztreonam was most affected by multiple substitutions. Aztreonam K_m values for all the enzymes bearing single mutations were essentially identical with the K_m for TEM-1, a value much higher than that observed for most β-lactam antibiotics. However, the K_m was decreased 23–56-fold in the mutant enzymes containing both serine and lysine substitutions. Improvement in binding to the enzyme with the triple mutations was observed only with ceftazidime.

All the enzymes were strongly inhibited by clavulanic acid. Although I_{50} values were lower for all the mutants containing the serine-164 substitution, the enzymes with double mutations had the highest apparent affinity for this well-described inactivator of the TEM-2 β-lactamase (Charnas et al., 1978). Although enzyme and clavulanate were preincubated for 5.0 min before substrate was added, some reversibility was observed as the reaction with cephaloridine proceeded in the presence of bound clavulanic acid. Further studies are necessary to determine whether a different mode of inactivation could be occurring in the mutant enzymes.

When catalytic efficiencies of the β-lactamases were compared (Table VI), it became quite evident that these enzymes were strong penicillinases, with benzylpenicillin hydrolyzed most efficiently of all the substrates in the study. Values for k_{cat}/K_m were not markedly affected for either cephaloridine or benzylpenicillin in the single lysine mutants and were de-

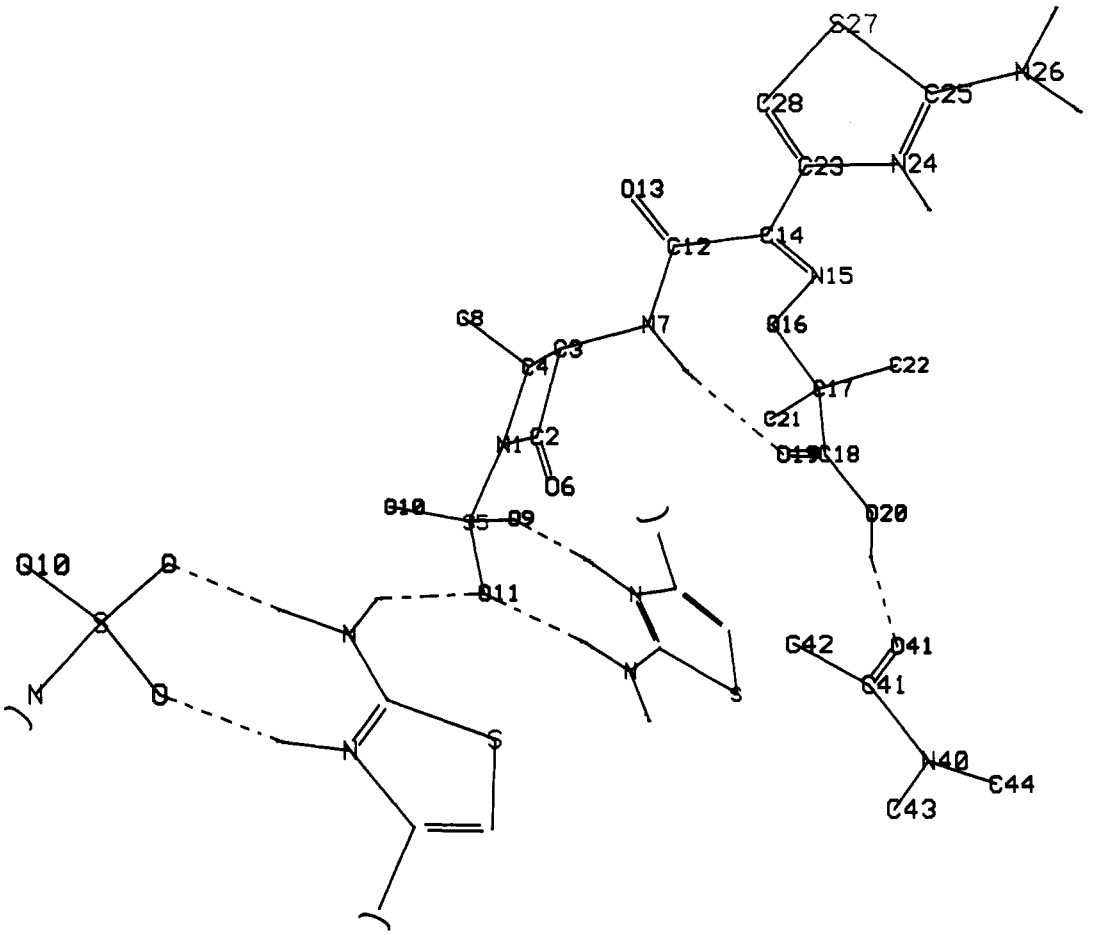

FIGURE 2: Hydrogen bonding in the crystal structure of aztreonam, **1**. Dimethylacetamide in the lattice is shown on the lower right. Partial structures in the lower half represent three neighboring monobactams.

creased less than 1 order of magnitude for all the mutants containing serine-164, compared to the TEM-1 enzyme. All the mutant enzymes exhibited higher hydrolysis efficiencies for the extended-spectrum antibiotics compared to the parent enzyme. Aztreonam and cefotaxime were similar when only the enzymes with single mutations were considered. However, aztreonam was much more efficiently hydrolyzed by the enzymes with double or triple mutations, due primarily to enhanced binding to these enzymes. Ceftazidime was the most dramatically affected antibiotic in this study. Substitution of either lysine resulted in more than a 20-fold increase in hydrolysis. The serine-164 replacement created an even more efficient enzyme for ceftazidime hydrolysis. However, the most impressive enzymes were those with double or triple mutations. These increases in k_{cat}/K_m values for ceftazidime must be due to increases in k_{cat}, not to improved binding as seen with the other cephalosporins and aztreonam.

Crystal Structure of Aztreonam and Related Derivatives. A precise description of the molecular structure of aztreonam, **1**, was obtained through single-crystal X-ray analysis of its dimethylacetamide-solvated (1:1) crystal structure (Figure 2). The antibiotic is zwitterionic: the anionic sulfonate group bears no hydrogen while the cationic aminothiazole group bears two hydrogens on the primary nitrogen, N26, and an additional hydrogen on the cyclic nitrogen, N24; O20 of the carboxylic acid also bears a hydrogen. All of these hydrogens are involved in intermolecular hydrogen bonds. The remaining hydrogen-bond donor, N7 of the side-chain amide group, is intramolecularly hydrogen-bonded to the carbonyl oxygen, O19, of the carboxylic acid. The amide carbonyl oxygens, O6 and O13, are not involved in hydrogen bonds.

The crystal structures of other monobactams were also studied in order to compare the intermolecular packing modes and conformations of their side chains. An intramolecular N7—O19═C—O hydrogen bond is also present in the crystal structure of the dicholine salt of the sulfate, **2**, but not in the dihydrated crystal structure of its zwitterionic form, **3**, where N7 is hydrogen-bonded to a water molecule. Both amide carbonyls of **3** are also hydrogen-bonded to water molecules.

Although these monobactams thus exhibit considerable variation in their modes of intermolecular crystal packing association, their side chains have similar conformations with respect to the plane, P1, of the side-chain amide group: the approximate plane P2 of the aminothiazole and oxime atoms (torsional angles N24–C23–C14–N15 = −13°, C23–C14–N15–O16 = −174°, C14–N15–O16–C17 = −157° in **1**) is rotated relative to P1 through torsional angle N7–C12–C14–N15 = 50–60° in **1**–**3** such that the aminothiazole ring and β-lactam carbonyl lie on the same side of P1, while the oxime atoms and atom C4 lie on the opposite side. The carboxyl group in all three structures is turned out of the plane P2 toward N7 (torsional angles N15–O16–C17–C18 fall in the range 75–84°). A superposition of the solid-state conformation of **1**–**3** is shown in Figure 3.

Discussion

TEM-1$_{pTZ18R}$ β-Lactamase. Plasmid pTZ18R is a phagemid derived from pBR322, a common source for TEM-1 β-lactamase. The choice of pTZ18R for mutagenesis studies was predicated on the necessity of being able to generate single-stranded DNA, which is essential for the mutagenesis protocol. Plasmid pTZ18R contains the f1 origin of replica-

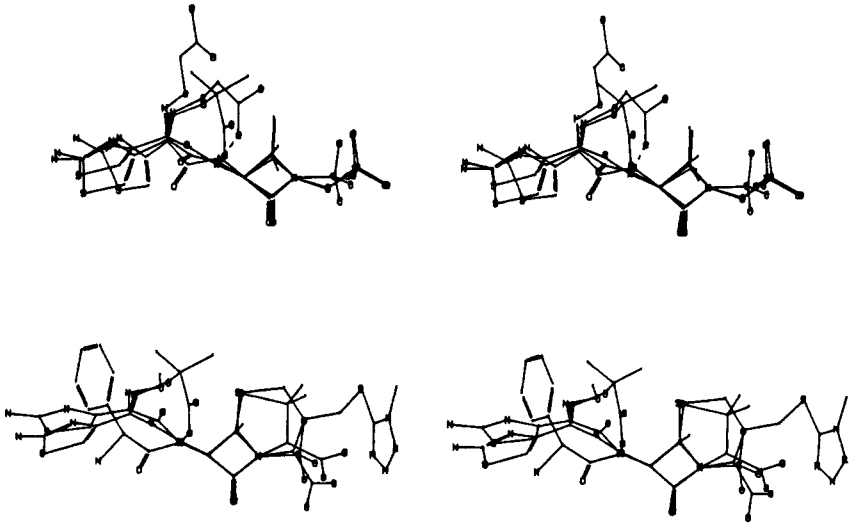

FIGURE 3: (Top) Superposition of the β-lactam rings in the crystal structures **1**–**3**. The side-chain amide plane P1 is approximately perpendicular to the plane of the drawing. (Bottom) Superposition of the β-lactam rings in the crystal structures of **1**, ampicillin (James et al., 1968), and cefmenoxime (Kamiya et al., 1981), a structure similar to cefotaxime.

tion, which allows one to infect cells with helper phage and generate quantities of single-stranded target DNA. The mutagenic primer is then annealed to this target DNA.

After desired mutations had been introduced, all the β-lactamase genes, in both the parent strain and the strains containing mutations, were sequenced in entirety. It was then discovered that the β-lactamase of pTZ18R had two additional base changes that differed from TEM-1, but these were remote from the residues postulated to be involved with the active site. Enzymatic properties of the parent pTZ18R β-lactamase were comparable to those obtained for an authentic TEM-1 enzyme.

β-Lactamase–Substrate Interactions. Many of the assumptions about specific residues involved in the active sites of TEM enzymes are based upon the crystal structures of the PC1 β-lactamase from *Staphylococcus aureus* (Herzberg & Moult, 1987) and the *B. licheniformis* β-lactamase (Moews et al., 1990), all members of class A β-lactamases (Ambler, 1980). β-lactamases have been described as "fully efficient" enzymes with first-order rate constants virtually identical for good substrates (Christensen et al., 1990). Hydrolysis of β-lactam antibiotics is accepted to proceed through a Michaelis complex to an acyl enzyme formed by way of a tetrahedral intermediate involving Ser-70 (Fisher et al., 1980). Deacylation of the enzyme is effected by a molecule of water and may involve interactions with Glu-166 (Herzberg & Moult, 1987). The water molecule available for the deacylation step has been proposed to lie at the bottom of the active-site depression with hydrogen bonding to the main-chain carbonyl of Gln-237, the side chain of Asn-170, and the carboxyl group of Glu-166.

Other active-site residues that have been proposed to be involved in hydrolysis of most substrates include Lys-234 that can interact with the carboxyl group on the 5- or 6-membered ring adjoining the β-lactam bond (Herzberg & Moult, 1987). Further evidence for involvement of Lys-234 as an electrostatic anchor has been provided by site-directed mutagenesis studies of Ellerby et al. (1990). Residue 235, glutamine in the PC1 enzyme, lies along the edge of the substrate-binding cavity following the triad of conserved residues beginning with Lys-234. Residue 237 has also been implicated in the active site, although it is not fully conserved among class A β-lactamases. It has been proposed that the amide backbone of this residue along with Ser-70 forms an oxyanion hole that may stabilize a tetrahedral intermediate similar to those proposed for well-characterized serine proteases (Healey et al., 1989). A conserved Lys-73 has been identified as critical in studies with the class A broad-spectrum β-lactamases as well as the class C cephalosporinases (Oefner et al., 1990; Tsukamoto et al., 1990).

Binding of the penicillin ampicillin to the PC1 enzyme also involves interactions of the positively charged ammonium side chain of the antibiotic. Herzberg and Moult (1987) have suggested that binding involves the side chains of Asn-170 and the main-chain carbonyl oxygen of Gln-237 and noted that antibiotics with bulkier substituents were less likely to be hydrolyzed. When novel class A β-lactamases began to be recognized in clinical infections, Labia et al. (1988) proposed that oxime-containing substrates were now being hydrolyzed because of an interaction between Ser-238 and the basic nitrogen of the oxime in the cephalosporin side chains. In addition, a second lysine residue other than that involved in binding the 4-carboxyl of the cephalosporin ring was suggested to be necessary for the hydrolysis of molecules such as ceftazidime that bear a second acidic group on the oxime side chain.

Three-Dimensional Structural Considerations. Since the three-dimensional atomic coordinates of the backbone and side-chain atoms of the active-site residues of the PC1 enzyme were not available, they were estimated from the measured parallax in the published stereodiagrams (Herzberg & Moult, 1987). The observed solid-state conformation of aztreonam was positioned in the active-site cleft by using the Herzberg–Moult model for the binding of ampicillin as a guide to the placement of the β-lactam ring. The qualitative placement was performed graphically on geometric grounds; no attempt was made to alter the observed conformation of aztreonam or to optimize the fit by energy-minimization techniques (Figure 4).

The β-lactam carbonyl points toward backbone atom N of Gln-237 and OG of Ser-70 lies on the α face of the β-lactam ring. Two of the anionic sulfonate oxygens are between OG of Ser-130 and NZ of Lys-234. The side-chain amide carbonyl points into the cleft toward the side chains of Asn-132 and Glu-166 while the N7–H bond is directed toward the backbone carbonyl oxygen of Gln-237. (In the unaltered conformation of aztreonam N7–H is intramolecularly hydrogen-bonded to the oxime side chain acid.)

As a consequence of these alignments, the thiazole ring in

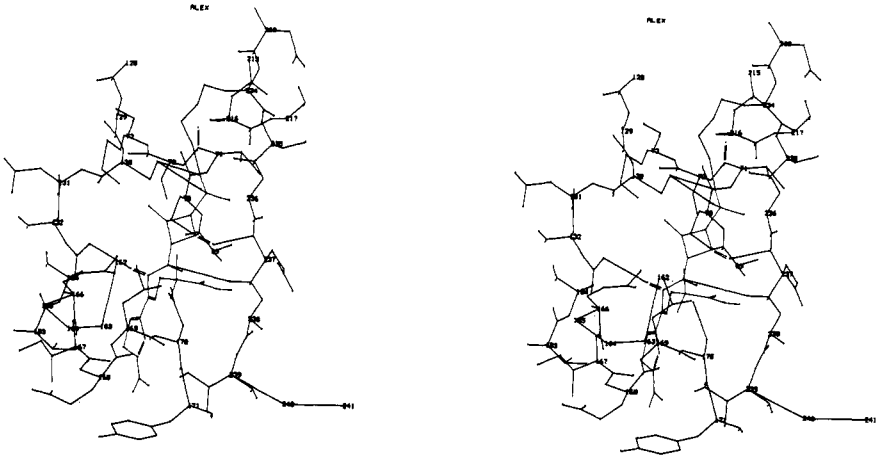

FIGURE 4: Stereorepresentation of an approximate model for the binding of aztreonam in the class A β-lactamase active site. The conformation of aztreonam is that observed in its own crystal structure.

plane P2 occupies a hollow of the cleft bounded by Ile-239 and Val-103–Ala-104, on opposite faces of the ring, and Ile-167 along the bottom of the hollow, in close contact with the S27–C28 edge of the ring. Primary amine N26 points toward the hydroxyl of Tyr-171. This hollow is the previously proposed binding site for the phenyl ring of ampicillin [the side-chain conformation was altered by Herzberg and Moult from that of the original crystal structure of ampicillin (see Figure 3) in order to achieve the fit].

The oxime atoms are exposed at the top of the cleft since plane P2 extends into the solvent, approximately bisecting the cleft opening between Val-103, Ala-104 on one side and Gln-237, Ala-238, Ile-239 on strand β3 on the other side. The observed positive rotation (75°) of the carboxyl out of P2 directs it toward the exposed side-chain amide of Gln-237. A similar though negative rotation out of P2 would direct it toward Ala-104 on the other side of the opening.

Interactions with Mutant β-Lactamases. An important sequence difference is found among the class A β-lactamases in the region 237–240: an insertion occurs at position 239 in the PC1 and *B. licheniformis* β-lactamases. We assume that, in three-dimensional space, residue 240 of the TEM enzymes occurs in a location similar to that of 239 in the PC1 enzyme, not position 240 as has been proposed in previous studies (Sougakoff et al., 1989). (A turn at 239 of the PC1 backbone extends 240 away from the active-site cleft; see Figure 4.) Lysines-104 and -240 of the mutant enzymes accordingly were substituted graphically for residues Ala-104 and Ile-239 in the above PC1–aztreonam model. It appears that electrostatic interactions of the oxime acid group of aztreonam or ceftazidime could be established with either lysine through rotations of the carboxyl group toward opposite sides of the cleft. Hydrogen-bonding interactions involving the N and O atoms of the oxime group and hydrogens on NZ of lysine also appear to be possible, particularly for the more proximate Lys-104, and may account for the enhanced kinetic properties of antibiotics, such as cefotaxime, that have no carboxyl group on the side chain. These ideas are consistent with the kinetic data that reveal similarly enhanced catalytic properties for either mutant enzyme containing a lysine/serine combination. There was little change in kinetic properties of the enzyme bearing three amino acid changes compared to the enzymes with double mutations, especially when compared to enzyme R164S, E240K.

Our experimental data indicate that binding of the various substrates is quite different when enzymes with multiple amino acid changes were examined. In the native enzyme and in those enzymes with single mutations, substrates with fused β-lactam structures bound much more effectively than the smaller monobactam. Binding to these enzymes probably is more affected by the β-lactam portion of the molecule. (Some adjustments of the fit of the β-lactam moiety to the enzyme clearly are necessary to compensate for the inherent geometric difference in the intramolecular separation of the β-lactam carbonyl and ionized acid group of penicillins, cephalosporins, and N-sulfonated monobactams.) Aztreonam exhibited a much greater affinity for those enzymes that had both serine and lysine substitutions, indicating that binding was enhanced only when the combination of changes occurred. K_m values for aztreonam binding to enzyme with multiple changes were comparable to those of cefotaxime and ceftazidime in the triple mutant. Thus, the side chain of the antibiotic must be a more important determinant for substrate affinity in these enzymes. These data suggest that multiple mutations may result in proteins that allow a better fit to the aminothiazole moiety.

The important role of Ser-164 is not understood. It is removed from the immediate vicinity of the active site, lying at the solvent boundary below residues 166–169, which delineate the bottom of the cleft hollow (Figure 5) (approximate Cα164–Cα*n* distances measured from the deposited Cα*n* coordinates of the PC1 enzyme: 6.8 Å (n = 166), 8.9 Å (167), 6.7 Å (168), 5.2 Å (169), 8.9 Å (170), 14.7 Å (239), 15.9 Å (104), 12.9 Å (70) (backbone and side-chain atoms below residue 166 were not shown in the active-site drawing of Herzberg and Moult). In the *B. licheniformis* enzyme the side chain of Arg-164 projects into the solvent (Moews et al., 1990), although its proximity to Glu-168 raises the possibility of a salt bridge in other enzymes. Since the single substitution R164S affects hydrolysis rates more dramatically than any other single change, it is possible that the configuration near the bottom of the cleft—a proposed site of a water molecule used in deacylation (Herzberg & Moult, 1987)—is somehow altered. The absence of arginine and not the presence of serine may be the basis for the observed differences. Arg-164 of the native enzyme is a residue conserved among the class A β-lactamases (Joris et al., 1988; Houba et al., 1989; Campbell et al., 1989) and is but two amino acids away from the totally conserved glutamic acid in box V of the penicillin-recognizing enzymes (Joris et al., 1988).

Many of the conclusions drawn from this study of TEM-derived enzymes are well supported by evidence from other laboratories. Our structural considerations have focused on the known conformation of aztreonam, and it is of interest that the orientation of the oxime acid group and the thiazole group

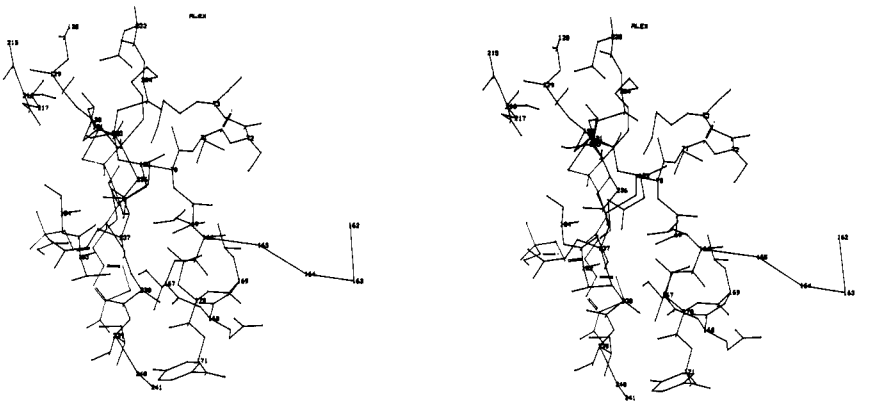

FIGURE 5: Stereorepresentation indicating the remote position of residue 164 in the model for aztreonam binding to a class A β-lactamase.

in the recently reported crystal structure of the acyl enzyme intermediate from aztreonam and the *Citrobacter freundii* class C β-lactamase (Oefner et al., 1990) is qualitatively consistent with our model.[1] However, other side-chain conformations of aztreonam cannot be excluded. Alternative side-chain conformations in which the oxime group and thiazole ring are spatially interchanged through rotations about the C12–C14 bond raise the possibility that interactions of the side-chain acid occur within rather than above the hollow. The side chains of Lys-104 and Lys-240 have considerable conformational flexibility for rotations either above or into the hollow toward Glu-266 (and Ser-164). We note that in the native PC1 enzyme, the Cα–Cβ bonds of residues 104 and 239 are directed into the hollow.

Future crystallographic studies of single and double mutant enzymes should clarify several of the unanswered questions raised in this study. Of fundamental concern here is the potential for significant local changes in protein structure when mutations, deletions, or insertions involve amino acids with large ionic side chains.

Evolutionary Considerations. The biological consequences of a single mutation may be relatively minor, as seen in the enzymes created by single lysine substitutions at either position 104 or 240. It is most likely that these changes and similar ones have occurred naturally in the TEM β-lactamases and have not been detected. Unless biological activity has been altered, single mutations would be difficult to identify.

In contrast, enzyme R164S, also the result of a single mutation, has a very different set of properties from the enzymes with single lysine mutations. This enzyme appears to be quite similar to TEM-101, an enzyme selected from pBR322 in *E. coli* grown in the presence of 2 μg/mL ceftazidime (Gutmann et al., 1988). Both of these enzymes have a single substitution of serine for arginine at position 164 (Collatz et al., 1989). Although the parent enzymes are not identical, the microbiological activities of parent and mutant strains are equivalent. MICs for cefotaxime and aztreonam differed by no more than 2-fold, whereas MICs for ceftazidime were elevated 8-fold for each of the serine mutants. Absolute hydrolysis rates for cefotaxime and ceftazidime were virtually identical for the R164S and TEM-101 enzymes (Gutmann et al., 1988). These results confirm the previous observations that the conservative mutations in TEM-1_{pTZ18} do not affect the biological parameters in a measurable manner.

However, the changes in biological properties of the producing organisms would not necessarily be observed unless a specific hydrolyzable substrate were used during clinical treatment. One can assume that a number of silent mutations, or minimally important mutations, occur frequently with little functional consequence. However, when plasmid-bearing bacteria are then exposed to an agent such as ceftazidime, a second mutation results in the growth of organisms resistant to this antibiotic.

Sougakoff et al. (1989) have described differences in nucleotide sequences between Tn3 and Tn2, two transposons coding for TEM-1 β-lactamases. Three silent mutations were identified in the coding region, and one mutation occurred in the promoter region. This kind of occurrence must be common and allows for bacteria to be ready for a more drastic change as a result of another point mutation when challenged with a previously stable antibiotic. Many of these point mutations alone are not detectable in the normal microbiological evaluation of organisms, even when amino acid substitutions occur. In this study enzymes carrying a single lysine substitution (E104K and E240K) were not easily identified on microbiological screening. However, acquisition of a single innocuous substitution may allow an enzyme to subsequently evolve to an enzyme with a more dangerous double mutation. The consequences of this have already been realized, as novel β-lactamases continue to be identified in bacteria causing life-threatening infections.

[1] The oxime acid extends above the active site. Three-dimensional coordinates of the cleaved antibiotic and the main-chain β strands 7 and 8 were estimated from the published stereodrawing of Figure 2b of Oefner et al. (1990). For the structural comparison, the α carbons of β strand 7 were superimposed with those of residues 236–239 of the PC1 β-lactamase.

Acknowledgments

We thank Ben Kolek for performing the microbiological testing of the mutant organisms. We especially thank Daniel P. Bonner and Robert Kessler for their support and critical comments related to these studies.

Supplementary Material Available

Tables giving unit cell data and atomic coordinates for **1–3** and a figure showing the atomic numbering scheme for **3** (11 pages). Ordering information is given on any current masthead page.

References

Ambler, R. P. (1979) in *Beta-Lactamases* (Hamilton-Miller, J. M. T., & Smith, J. T., Eds.) pp 99–125, Academic Press, London.

Ambler, R. P. (1980) *Philos. Trans. R. Soc. London, B 289*, 321–331.

Ambler, R. P., & Scott, G. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3732–3736.

Bush, K. (1989) *Antimicrob. Agents Chemother. 33*, 264–270.
Bush, K., & Singer, S. B. (1989) *J. Antimicrob. Chemother. 24*, 82–84.
Campbell, J. I. A., Scahill S., Gibson, T., & Ambler, R. P. (1989) *Biochem. J. 260*, 803–812.
Cartwright, S. J., & Waley, S. G. (1984) *Biochem. J. 221*, 505–512.
Chanal, C., Sirot, D., Labia, R., & Sirot, J. (1989) *J. Antimicrob. Chemother. 24*, 86–87.
Charnas, R. L., Fisher, J., & Knowles, J. R. (1978) *Biochemistry 17*, 2185–2189.
Christensen, H., Martin, M. T., & Waley, S. G. (1990) *Biochem. J. 266*, 853–861.
Collatz, E., Tran Van Nhieu, G., Billot-Klein, D., Williamson, R., & Gutmann, L. (1989) *Gene 78*, 349–354.
Dalbadie-McFarland, G., Cohen, L. W., Riggs, A. D., Morin, C., Itakura, K., & Richards, J. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6409–6413.
Dube, D. K., & Loeb, L. A. (1989) *Biochemistry 28*, 5703–5707.
Ellerby, L. M., Escobar, W. A., Fink, A. L., Mitchinson, C., & Wells, J. A. (1990) *Biochemistry 29*, 5797–5806.
Fisher, J., Belasco, J. G., Khosla, S., & Knowles, J. R. (1980) *Biochemistry 19*, 2895–2901.
Gutmann, L., Kitzis, M. D., Billot-Klein, D., Goldstein, F., Tran Van Nhieu, G., Lu, T., Carlet, J., Collatz, E., & Williamson, R. (1988) *Rev. Infect. Dis. 10*, 860–866.
Hall, A., & Knowles, J. R. (1976) *Nature (London) 264*, 803–804.
Healey, W. J., Labgold, M. R., & Richards, J. H. (1989) *Proteins 6*, 275–283.
Herzberg, O., & Moult, J. (1987) *Science 236*, 694–701.
Houba, S., Willem, S., Duez, C., Molitor, C., Dusart, J., Frere, J.-M., & Ghuysen, J.-M. (1989) *FEMS Microbiol Lett. 65*, 241–246.
James, M. N. G., Hall, D., Hodgkin, D. C., (1968) *Nature 220*, 186.
Joris, B., Ghuysen, J.-M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frere, J.-M., Kelly, J. A., Boyington, J. C., Moews, P. C., & Knox, J. R. (1988) *Biochem. J. 250*, 313–324.
Kamiya, K., Takamoto, M., Wada, Y., & Nishikawa, M. (1981) *Chem. Pharm. Bull. 29*, 609.
Kunkel, T. A. (1987) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) Greene Publishing Associates & Wiley Interscience, New York.
Labia, R., Morand, A., Tiwari, K., Sirot, J., Sirot, D., & Petit, A. (1988) *Rev. Infect. Dis. 10*, 885–891.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Mabilat, C., Goussard, S., Sougakoff, W., Spencer, R. C., & Courvalin, P. (1990) *Plasmid 23*, 27–34.
Medeiros, A. A. (1984) *Brit. Med. Bull. 40*, 18–27.
Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., & Frere, J.-M. (1990) *Proteins 7*, 156–171.
National Committee for Clinical Laboratory Standards (1985) *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard M7-A*, National Committee for Clinical Laboratory Standards, Villanova, PA.
Oefner, C., D'Arcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., Heinze, I., Hubschwerfen, C., & Winkler, F. K. (1990) *Nature 343*, 284–288.
Peduzzi, J., Barthelemy, M., Tiwari, K., Mattioni, D., & Labia, R. (1989) *Antimicrob. Agents Chemother. 33*, 2160–2163.
Philippon, A., Labia, R., & Jacoby, G. (1989) *Antimicrob. Agents Chemother. 33*, 1131–1136.
Quinn, J. P., Miyashiro, D., Sahm, D., Flamm, R., & Bush, K. (1989) *Antimicrob. Agents Chemother. 33*, 1451–1456.
Rossmann, M. G., & Argos, P. (1980) *Acta Crystallogr. B36*, 819–823.
Schultz, S., & Richards, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1588–1592.
Sigal, I. S., Harwood, B. G., & Arentzen, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7157–7160.
Sougakoff, W., Goussard, S., & Courvalin, P. (1988) *FEMS Microbiol. Lett. 56*, 343–348.
Sougakoff, W., Petit, A., Goussard, S., Sirot, D., Bure, A., & Courvalin, P. (1989) *Gene 78*, 339–348.
Sutcliffe, J. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3737–3741.
Tsukamoto, K., Tachibana, K., Yamazaki, N., Ishii, Y, Ujiie, K., Nishida, N., & Sawai, T. (1990) *Eur. J. Biochem. 188*, 15–22.